Genetically Modified Plants for Improved Trace Element Nutrition[1]

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ABSTRACT Deficiencies of iron and zinc are common worldwide. Various strategies have been used to combat these deficiencies including supplementation, food fortification and modification of food preparation and processing methods. A new possible strategy is to use biotechnology to improve trace element nutrition. Genetic engineering can be used in several ways; the most obvious is to increase the trace element content of staple foods such as cereals and legumes. This may be achieved by introduction of genes that code for trace element–binding proteins, overexpression of storage proteins already present and/or increased expression of proteins that are responsible for trace element uptake into plants. However, even very high levels of expression may not substantially increase the iron and zinc contents unless many atoms of trace elements are bound per protein molecule. Another possibility is to introduce a protein that specifically enhances trace element absorption even in the presence of naturally occurring inhibitors, thus improving bioavailability. Genetically modifying plants so that their contents of inhibitors of trace element absorption such as phytate are substantially reduced is another approach. Increasing the expression of compounds that enhance trace element absorption such as ascorbic acid is also a possibility, although this has received limited attention so far. Iron absorption may be increased by higher ascorbic or citric acid content but require overexpression of enzymes that are involved in the synthetic pathways. Finally, a combination of all of these approaches perhaps complemented with conventional breeding techniques may prove successful. J. Nutr. 133: 1490S–1493S, 2003.

KEY WORDS: • *genetically modified plants* • *gene insertion* • *plant breeding* • *trace element absorption* • *bioavailability* • *iron* • *zinc*

It is now well recognized that iron deficiency is a global problem and that suboptimal zinc nutrition is more common than previously believed. Many programs have been instituted to both prevent and treat iron and zinc deficiency that range from supplementation and fortification of common staple foods to modification of traditional food-preparation methods (to optimize bioavailability). All of these methods have value, but infrastructure, economics, local habits and availability of centralized facilities and health care professionals can limit their utility. It is therefore reasonable to attempt to use all approaches available to improve the iron and zinc nutrition of populations in need.

Genetically modifying plants for improved trace element nutrition is a complementary approach. By increasing the trace element content of traditionally grown crops and/or enhancing the bioavailability of iron and zinc in such crops, trace element nutrition of the population that consumes these crops may be improved. Genetic modification can be achieved by two fundamentally different approaches. First, by using conventional breeding and selection techniques, cultivars with the highest content of trace elements, the highest concentration of enhancers of trace element bioavailability and/or the lowest content of inhibitors of trace element absorption can be bred into stable and high-producing lines. Second, genetic engineering techniques can be used to create novel cultivars with the desired properties. Examples of such approaches are the insertion of novel genes, enhancement of the expression of genes already present but at low expression levels, and depression of the expression of genes or disruption of pathways involved in the synthesis of inhibitors of trace element absorption. In this overview, I attempt to organize these different approaches into four major areas. It should be noted that there is no need to select any of these approaches; it is quite possible to use them in combination to optimize trace element bioavailability.

Increasing the trace element content of plants

Insertion of genes for novel metal-binding proteins. To date, two novel iron-binding proteins have been incorporated into rice. Rice has several advantages as compared to other

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plants: rice proteins have very low allergenicity (i.e., any contaminants from rice in products such as infant formula or baby foods are unlikely to cause adverse reactions), rice contains no toxic compounds and very high expression levels can be achieved.

We have expressed human lactoferrin, the major iron-binding protein in breast milk, in rice (1,2). By using the so-called gene-gun technology with a strong endosperm-specific promoter from rice and selecting for high-lactoferrin cultivars, very high expression levels (5 g of lactoferrin/kg of grain; 6% of total protein) could be achieved. Gene expression has been stable for more than five generations, and large-scale field trials have yielded harvests at levels of several tons. Each molecule of lactoferrin binds two atoms of ferric iron (3), and the recombinant lactoferrin is shown to be fully iron saturated. This doubles the iron content of the dehusked (polished) rice but has no effects on the contents of other nutrients (2). However, although the iron content is increased by 120%, this increase is very modest (1 mg of iron/100 g of dry matter) and will not provide a substantial enhancement of the dietary iron intake of adults even if rice is a major part of the staple food. We have shown that human lactoferrin binds specifically to receptors in the human small intestine (4,5) and this fact, as well as that iron is bound in the ferric form, makes it unlikely that inhibitors of iron absorption such as phytate will inhibit iron absorption from lactoferrin. However, even if the bioavailability of iron from lactoferrin can be expected to be high, the quantity of iron absorbed will be limited. For infants and young children, whose total iron requirement is relatively low, this may be a valuable addition to their daily absorbed iron, but for adults this may be relatively insignificant.

It is thus likely that insertion of a gene for a protein that can bind more metal ions per molecule is needed to achieve a trace element content that is high enough to meet the iron requirements of adults. Ferritin is such a protein: each ferritin molecule can bind as many as 4,500 atoms of iron (6). The gene for soybean ferritin has been inserted to rice (7). These experiments seem to have been done on a smaller scale, but the iron content in a few transformants is two- to threefold that of wild-type. Iron in this type of transgenic rice also appears to be well utilized in iron-depleted rats (8). Another research group inserted the ferritin gene from *Phaseolus vulgaris* into rice (9). By using *Agrobacterium*-mediated transformation and the rice glutellin promoter, they were able to double the iron content of dehusked rice. Considering the very high iron-binding capacity of ferritin, however, this iron concentration is somewhat disappointing. It is possible that these experiments, which appear to have been done on a relatively limited scale, can be extended to increase the expression level of ferritin. It is also possible, however, that expression levels of ferritin were high (which is difficult to evaluate from the Western blots), but that the plant's system of delivering iron to the rice seeds was inadequate to achieve higher iron levels (see "*Expression of transporters that facilitate plant uptake of trace elements*"). At present, the iron content of rice that expresses ferritin does seem similar to that of rice that expresses lactoferrin, i.e., it is unlikely to be adequate to substantially increase the iron intake of adults.

The bioavailability of ferritin iron may be high. Although early experiments indicate low iron absorption from ferritin, those experiments are flawed, and recent studies suggest that ferritin iron may be as available as iron from ferrous sulfate (8,10). Potential concerns with high-level expression of ferritin in rice include the color of the rice (modest change at low levels; high levels?), which may affect consumer attitude and allergenicity.

Increased grain content by trace element fertilizers. Although there are some soils that are deficient in trace elements that may benefit from trace element fertilizers, under most conditions there appears to be some biological limit that makes it difficult to increase the concentration of trace elements such as iron and zinc in grains and legumes. Selenium, however, is an exception: the selenium content of plants is directly correlated to soil selenium; thus, trace element fertilization has a considerable impact on crop selenium contents. This is used in Finland, a country with low levels of selenium in the soil and the food supply, where selenium fertilization is successfully applied to improve the selenium status of the population.

Overexpression of "native" genes for trace element storage proteins. There are trace element–binding proteins in plants that are expressed either at low levels or in a plant tissue that is normally not eaten. One example of the first category is soybean ferritin, which is present in the soy endosperm, but the level of expression is too low to contribute substantially to the soybean iron content. By either selecting and breeding for high-ferritin varieties or (which is most likely needed to achieve high-expression levels) overexpressing the soybean ferritin gene, it should be possible to increase substantially the ferritin content of soy. Similarly, most plants do contain ferritin, e.g., rice, and such an approach may be used. Whether the increased ferritin expression is correlated to a concomitant increase in iron content is likely to be dependent on the plant's capacity to take up iron and transport it to the endosperm (see *Expression of transporters that facilitate plant uptake of trace elements*).

An example of a trace element–binding protein that is present in the "wrong" tissue of the plant (from the consumer's perspective) is leghemoglobin (from legume-hemoglobin) (11). This protein is present in the root nodules of legumes such as soybeans and accumulates iron in the heme form. Because absorption of iron from heme is high and also is not affected by inhibitors of iron absorption (12), this may be a useful form of iron in human nutrition. Preliminary experiments on rats indicate a high bioavailability of iron from soybean leghemoglobin (Boy et al., personal communication), but further experiments on humans are needed to verify this. Whether the expression of leghemoglobin can be directed to the seeds (beans) or whether the gene can be inserted as a storage protein in other plants such as rice or cereals needs to be explored. Attempts to insert the soybean leghemoglobin-encoding gene into potato tubers resulted in reduced growth and decreased tuber production compared with untransformed plants (13), which suggests that redirection of this gene into other parts of a plant may be difficult.

Expression of transporters that facilitate plant uptake of trace elements. As stated previously, the uptake of trace elements by the root system of plants and the accumulation of trace elements by the seed or grain is likely to be limited by the capacity of the plant to take up and transport these ions (14). First, the mineral ions need to be available at and pass the root-soil interface; this must be facilitated to substantially increase the trace element content of plants. Next, the absorption mechanisms in the root-cell plasma membrane need to be sufficient and adequately specific to allow the trace elements to accumulate once they have entered the apoplasm, i.e., the space between cells. Finally, the trace elements must be efficiently transported to the edible parts of the plant. Phloem sap loading, translocation and unloading rates are important processes that need to be considered. Plant biologists have identified and characterized the proteins and genes involved in these transport processes (15). Experiments are now needed in which key transporters are overexpressed and seed accumulation of trace elements such as iron and zinc is studied. In all of these studies, it is a given that plant viability, crop yield, disease resistance, etc. are studied, as there may be a limit beyond which overaccumulation of trace elements may be toxic to the plant or impair the plant's normal functions. It was also shown that it can be difficult to direct the overexpression of genes to the proper part of the plant (16).

Expression of proteins that facilitate trace element uptake

Certain proteins can survive digestion in intact or partially intact form and are therefore capable of facilitating trace element absorption in the small intestine. Lactoferrin and ferritin, which are described earlier as "storage" proteins for iron, are also capable of facilitating trace element uptake. Lactoferrin is remarkably resistant against proteolytic enzymes, and intact lactoferrin is detected in biologically significant quantities in the stool of breastfed infants. Lactoferrin has a very high affinity for iron and does not release iron until the pH is < 3. Even if the gastric pH in adults is likely to become < 3 and iron may be released from lactoferrin, the high binding affinity for iron and the combination with pancreatic bicarbonate, which facilitates the binding of iron to lactoferrin, is likely to result in reformation of holo-lactoferrin (3). Human lactoferrin is shown to bind to specific receptors on the surface of the brush-border membrane of the intestinal cell (4) with concomitant iron uptake by the cell (5). This may be particularly relevant for infants in whom the gastric pH is much higher than in adults and pepsin and pancreatic enzyme activities are low (17). We have shown that human lactoferrin can survive digestion and is found intact in the stool of breastfed infants (18), which originally made us suggest to express recombinant human milk proteins in various systems (19). Recently, lactoferrin was expressed in potatoes (20), although the rationale for their study is the antimicrobial properties of this protein.

There are also indications that ferritin can facilitate iron uptake in the small intestine. Experiments on rats show that hemoglobin regeneration in anemic rats is similar for soybean ferritin and ferrous sulfate (10), and recent human studies also suggest good utilization of iron from soybean ferritin (21). The mechanism by which iron is taken up from ferritin is not yet known, but our preliminary in vitro experiments in Caco-2 cells suggest that digestion of ferritin is limited and that subunits or other larger fragments of ferritin may be taken up by the cell with iron (Kelleher et al., unpublished data). These fragments are subsequently digested by some intracellular compartment and release iron for further transport into the circulation. Further experiments are needed to better delineate these processes.

Decreased inhibitors of trace element absorption

Low-phytate varieties. Phytic acid is an inhibitor of iron and zinc absorption in humans and is believed to be a major contributing factor to the worldwide problem of iron and zinc deficiency (22). Decreasing the phytate content of the diet is shown to be strongly correlated to increased iron (23) and zinc absorption (24). Thus, any reduction in the content of phytate in staple foods is likely to result in improved iron and zinc status.

Selective breeding for low-phytate varieties of several staple crops was recently shown to be successful (25). Spontaneous low–phytic acid (*lpa*) mutations have been found in maize, barley and rice and result in seed phytic acid–phosphorus levels that range from 50 to $> 95\%$ of controls (25,26). These plants have normal levels of total phosphorus but significantly reduced levels of phytic acid, which in turn increases the level of inorganic phosphorus. These high levels of inorganic phosphorus provide a quick, sensitive and inexpensive test for this trait and thereby make plant breeding practical (27). The first *lpa* mutation (*lpa* 1-1) in maize was introduced into several inbred lines using traditional backcrossing breeding techniques (25). Most work to date has been done on this maize mutant. We investigated iron absorption in humans from test meals based on tortillas baked with regular maize or *lpa* maize and found significantly increased iron absorption from the low-phytate variety (28,29).

Experiments on suckling rat pups showed that *lpa* varieties also enhance zinc absorption as compared to wild-type varieties (Lönnerdal et al., unpublished data). Fasted rat pups were intubated with suspensions of *lpa* varieties of maize, rice and barley, and tissue distribution of ^{65}Zn as well as whole-body retention were determined. The *lpa* varieties significantly increase intestinal and liver uptake of ^{65}Zn and whole-body retention of ^{65}Zn, which indicates that the reduction in phytate content is likely to have a positive effect on zinc nutrition in populations that consume these varieties. Human studies, both single-meal studies and long-term field trials, are needed to evaluate this.

Insertion of phytase gene. It was shown in both animal and human studies that exogenous or endogenous phytase can reduce the phytate content of the diet and improve trace element bioavailability (24,30,31). For example, phytase in grains can be allowed to act during the leavening process to substantially lower the phytate content of bread (32). Microbial phytase can also be added to feeds and allowed to act during the digestive process to improve mineral utilization in domestic animals (33,34).

Another possibility is to insert a gene for phytase into staple crops and express it at high levels. This approach has been attempted by Lucca et al. (9), who inserted a fungal (*Aspergillus fumigatus*) phytase into rice. This phytase was chosen because it is heat stable and therefore expected to remain active after food processing such as cooking. They achieved high expression levels of phytase in rice, but unfortunately, boiling the rice destroyed the phytase activity and only a very modest reduction in phytate was obtained. It is possible that another phytase that is more resistant against boiling can be used, which will possibly result in a more substantial reduction in phytate. Recent discoveries of extremely thermophilic microorganisms may aid in finding such a phytase. However, the potential allergenicity of such species-foreign proteins must be investigated in detail.

Increased synthesis of enhancers of trace element absorption

Amino acids. Various dietary ligands can enhance trace element absorption. One example of this is amino acids, which are largely released from proteins during digestion. Cysteine or cysteine-rich peptides are shown to have a positive effect on iron absorption (35,36) and histidine is shown to facilitate zinc absorption (37). Other organic acids such as fumarate, citrate and succinate can also enhance trace element absorption.

As an approach to increasing the cysteine content of the diet, Lucca et al. (9) inserted the gene for a rice metallothionein-like protein. This protein is normally induced during stress, and similar to metallothionein, it contains a high percentage of cysteine residues: 12 residues/mol of protein (38). Overexpression of this gene in rice results in a very high cysteine content (10 times higher than normal). The long-term consequences for the plant of this high level of expression of the metallothionein-like protein are not known, and the effect on iron absorption in humans has not yet been studied, but these early results are encouraging. It may be cautioned that even if this protein is not induced by metal ions (unlike metallothionein), it is not known whether high-level expression can cause accumulation of toxic metal ions.

Vitamins. There are reports in the literature that β-carotene can enhance iron absorption in humans (39), although the mechanism behind this effect is unknown. It is possible that β-carotene has a direct stimulatory effect on iron uptake by the cell, presumably by some complex being formed, or that β-carotene "conditions" the intestinal mucosa and therefore has long-term effects on iron absorption. Whether the so-called "golden rice" that has high levels of β-carotene (40) enhances iron absorption or status in humans is not yet known, but experiments are under way to resolve this.

One of the best-known enhancers of iron absorption is ascorbic acid (23). Many studies show that increasing dietary ascorbic acid leads to a substantial increase in iron absorption in humans. Thus, overexpression of ascorbic acid in plants is likely to have a positive effect on iron nutrition of human populations. High expression levels of α-tocopherol and β-carotene were achieved in plants (40,41), but there are limited reports on water-soluble vitamins. Most synthetic pathways for vitamins are quite complex, and it is possible that both insertion of novel genes as well as promoters for enzymes involved in these pathways are required.

In conclusion, it can be expected that significant advances will be made in the near future with regard to establishing genetically modified plants with increased contents of trace elements and optimized composition of enhancers and inhibitors of trace element absorption. Much further work is needed to prove that this will lead to healthy plants with maintained crop yield and that the trace element status will be enhanced in populations that consume these new varieties as part of their staple foods. Acceptability and safety issues need to be considered carefully, and property rights and economic implications must be solved before this can lead to a sustainable improvement in trace element nutrition.

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